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THE

AMERICAN NATURALIST

VOL. XVI. — *SEPTEMBER*, 1882. — NO. 9.

METHODS OF MICROSCOPICAL RESEARCH IN THE ZOOLOGICAL STATION IN NAPLES.

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IN the preparation of this paper Dr. Mayer has allowed me to make free use of his excellent article,[1] published about two years ago. I have added the methods of Dr. Giesbrecht, Dr. Andres and some others who have worked in the zoölogical station. Dr. Mayer has further placed at my disposal such improvements and alterations as he has been able to make since the publication of his paper. I am also deeply indebted to Dr. Mayer for advice and generous assistance, for which I wish here to give expression to my most sincere thanks and grateful appreciation.

I am still further indebted to Dr. Eisig, Dr. Lang, Dr. Andres, Dr. Giesbrecht, Professor Weismann and Professor Dohrn, all of whom I have had occasion to consult with reference to matter contained in this paper.

I. PRESERVATIVE FLUIDS.

Killing, *hardening* and *preserving* are three kinds of work, requiring for their accomplishment sometimes only a single preservative fluid, but in most cases two, three or even more. As the same fluid often does the work of killing and hardening, and sometimes of preserving too, it is impossible to divide them into three classes corresponding to the kinds of work, except by repeating many of them twice, and some of them three times. While it is therefore more convenient to include them all under "preservative fluids," as Dr. Mayer has done, it is none the less important to remember what kind or kinds of work each fluid is expected to accomplish.

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[1] Mayer. "Mittheilungen aus der Zoologischen Station zu Neapel." Vol. II, p. 1, 1880. A summary of this paper by Geo. Brook was published in the NATURALIST, June–Oct., 1881.

Kleinenberg's picro-sulphuric acid, for instance, now so much used in the Naples Aquarium, is not a hardening fluid. It serves for killing, and thus prepares for subsequent hardening.

1. *Kleinenberg's Fluid.*[1]—

Picric acid (saturated solution in distilled water)	100 volumes.
Sulphuric acid (concentrated)	2 "

Filter the mixture and dilute it with *three* times its bulk of water;[2] finally add as much creosote[3] as will mix.[4]

Objects are left in the fluid three, four or more hours; and are then, in order to harden and remove the acid, transferred to 70 per cent. alcohol, where they may remain 5–6 hours. They are next placed in 90 per cent. alcohol, which must be changed at intervals until the yellow tint has wholly disappeared.

Summary of Dr. Mayer's remarks on Kleinenberg's Fluid.—The advantages of this fluid are, that it kills quickly, by taking the place of the water of the tissues; that it frees the object from sea-water and the salts contained in it, and that having done its work *it may be wholly replaced by alcohol.* In this latter fact lies the superiority of the fluid over *osmic* and *chromic* solutions, all of which produce inorganic precipitates and thus leave the tissues in a condition unfavorable to staining. Picro-sulphuric acid does not, like chromic solutions, harden the object, but simply kills the cells.

As this fluid penetrates thick *chitine* with difficulty, it is necessary, in order to obtain good preparations of larger Isopoda, insects, &c., to cut open the body and fill the body-cavity with the liquid by means of a pipette. In larger objects care should be taken to loosen the internal organs so that the fluid may find easy access to all parts.

The fluid should be applied as soon as the body is opened, so that the blood may not have time to coagulate and thus bind

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[1] *Quart. Journ. Mic. Sci.*, Vol. XIX, p. 208–9, 1879.

[2] Dr. Mayer uses the fluid undiluted for Arthropoda.

[3] Creosote made from beechwood tar.

[4] Dr Mayer prepares the fluid as follows:

Water (distilled)	100 volumes.
Sulphuric acid	2 "
Picric acid (as much as will dissolve).	

Filter and dilute as above. No creosote is used.

the organs together. *A large quantity of the fluid should be used, and it must be changed as often as it becomes turbid.* The same rule holds good in the use of all preservative fluids. It is well also, especially with larger objects, to give the fluid an occasional stirring up.

In order to avoid shrinkage in removing small and tender objects from the acid to the alcohol, it is advisable to take them up by means of a pipette or spatula, so that a few drops of the acid may be transferred along with them. The objects, sinking quickly to the bottom, remain thus for a short time in the medium with which they are saturated, and are not brought so suddenly into contact with the alcohol. In a few minutes the diffusion is finished; and they may then be placed in a fresh quantity of alcohol, which must be shaken up frequently and renewed from time to time until the acid has been entirely removed.

The sulphuric acid contained in this fluid causes *connective tissue* to swell, and this fact should be borne in mind in its use with vertebrates. To avoid this difficulty Kleinenberg has recommended the addition of a few drops of creosote, made from beechwood tar, to the acid. According to Dr. Mayer's experience, however, the addition of creosote makes no perceptible difference in the action of the fluid.

This fluid must not be used with objects (*e. g.*, Echinoderms) possessing calcareous parts which it is desired to preserve, for it dissolves carbonate of lime and throws it down as crystals of gypsum in the tissues. For such objects *picro-nitric acid* may be used. It is prepared as follows:

Water	95 parts.
Nitric acid (25 per cent. N_2O_5)	5 "
Picric acid as much as will dissolve.[1]	

Picro-nitric acid also dissolves carbonate of lime, but it holds it in solution, and thus the formation of crystals of gypsum is avoided. In the presence of much carbonate of lime, the rapid production of carbonic acid is liable to result in mechanical injury of the tissues, hence in many cases chromic acid is preferable to picro-nitric acid.

Picro-nitric acid is, in most respects, an excellent preservative medium, and as a rule will be found to be a good alternative in those cases where picro-sulphuric acid fails to give satisfactory results. Dr. Mayer commends it very strongly, and states that with eggs containing a large amount of yolk material, like those of Palinurus, it gives better results than nitric, picric or picro-sulphuric acid. It is not so readily removed from objects as picro-sulphuric acid, and for this reason the latter acid would be used wherever it gives equally good preparations.

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[1] This mixture is used undiluted.

2. *Alcohol.*—In the preparation of animals or parts of animals for museums or histological study, it is well known that the chief difficulties are met in the process of killing. Alcohol, as *commonly* used for this purpose by collectors, has little more than its convenience to recommend it. Dr. Mayer has called attention to the following disadvantages attending its use in the case of marine animals:

(1) In thick-walled animals, particularly those provided with chitinous envelopes, alcohol causes a more or less strong maceration of the internal parts, which often ends in putrefaction.

(2) In the case of smaller Crustacea, *e. g.*, Amphipods and Isopods, it gives rise to precipitates in the body-fluids, and thus solders the organs together in such a manner as often to defy separation even by experienced hands.

(3) It fixes most of the salts of the water adhering to the surface of marine animals, and thus a crust is formed which prevents the penetration of the fluid to the interior.[1]

(4) This crust also prevents the action of staining fluids, except aqueous solutions, by which it would be dissolved.

Notwithstanding these drawbacks alcohol is still regarded at the Naples Aquarium as an excellent fluid for *killing* many animals designed for preservation in museums or for histological work. In many cases the unsatisfactory results obtained are to be attributed not to the alcohol *per se*, but to the *method* of using it. Most of the foregoing objections do not, as Dr. Mayer has expressly stated, apply to fresh-water animals; and Dr. Eisig informs me that he has no better method of killing marine annelids than with alcohol. Judging from the preparations which were kindly shown to me, and which were all beautifully stained with

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[1] Dr. Mayer first noticed this in objects stained with Kleinenberg's hæmatoxylin, and afterwards in the use of cochineal, where a gray-green precipitate is sometimes produced which renders the preparation worthless. Such results may be avoided by first soaking the objects a few hours in *acid alcohol* (1–10 parts hydrochloric acid to 100 parts 70 per cent. alcohol).

borax-carmine, Dr. Eisig's mode of treatment must be pronounced very successful. The process is extremely simple; a few drops of alcohol are put into a vessel which contains the annelid in its native element, the sea-water; this is repeated at short intervals until death ensues. After the animal has been thus slowly killed, it may be passed through the different grades of alcohol in the ordinary way, or through other preservative fluids. Objects killed in this manner show no trace of the external crust of precipitates which arises where stronger grades of alcohol are first used. The action of the alcohol is thus moderated, and the animal, dying slowly, remains extended and in such a supple condition that it can easily be placed in any desired position. The violent shock given to animals when thrown alive into alcohol of 40 per cent. to 60 per cent., giving rise to wrinkles, folds and distortions of every kind, is thus avoided, together with its bad effects.

3. *Acid Alcohol.*—In order to avoid the bad effects of alcohol, such as precipitates, maceration, &c., Dr. Mayer recommends *acid alcohol*—

95 volumes 70 per cent. or 90 per cent. alcohol.
3 " hydrochloric acid.[1]

for larger objects, particularly if they are designed for preservation in museums. The fluid should be frequently shaken up, and the object only allowed to remain until thoroughly saturated, then transferred to pure 70 per cent. or 90 per cent. alcohol, which should be changed a few times in order to remove all traces of the acid. For small and tender objects, acid alcohol, although preferable to pure alcohol, gives less satisfactory results than picro-sulphuric acid.

4. *Boiling Alcohol.*—In some cases among the Arthropods, Dr. Mayer has found it difficult to kill *immediately* by any of the ordinary means, and for such cases recommends *boiling absolute alcohol*, which kills instantly. For Tracheata this is often the only means by which the dermal tissues can be well preserved, as cold alcohol penetrates too slowly.

5. *Osmic Acid.*—Dr. Mayer employs osmic acid as a staining medium for the hairs, bristles, &c., of the dermal skeleton of Arthropods. The luster of Sapphirina is preserved by this acid,[2]

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[1] Acid alcohol as above prepared loses its original qualities after standing some time, as ether compounds are gradually formed at the expense of the acid.

[2] See corrosive sublimate, p. 705.

and according to Emery, the color of the red and the yellow fatty pigments of fishes.

Van Beneden found osmic acid the best preservative fluid for the Dicyemidæ, and my experience leads to the same conclusion.[1]

Although Dr. Mayer seldom uses this medium where histological details are required, he observes that in those classes of animals whose bodies are easily penetrated with watery fluids, osmic acid is seldom to be dispensed with.

Bleaching.—It often happens that objects treated with osmic acid continue to blacken, after removal from the acid, until they are entirely worthless, and such results are even more annoying than the difficulties in the way of staining. It has been said that the blackening process can be arrested by certain staining media, but it is certain that picro-carmine will not always do this, as some of my preparations of Dicyemidæ show. It is therefore a very important step which Dr. Mayer has taken in finding a method of restoring such objects. The method[2] is as follows: *The objects are placed in 70 per cent. or 90 per cent. alcohol, and crystals of potassic chlorate ($KClO_3$) shaken into the liquid until the bottom of the vessel is covered; then a few drops of concentrated hydrochloric acid[3] are added with a pipette, and as soon as chlorine (easily recognized by its greenish-yellow color) begins to be liberated, the whole gently shaken. As soon as the bleaching is finished the objects are removed to pure alcohol.* By this method Dr. Mayer has been able in half a day to restore large Pelagia, Carinaria, Rhizostoma, &c. Small objects generally require a shorter time and less acid. The process can be greatly accelerated by heating on a water-bath.

Using Sapphirina as a test-object, Dr. Mayer found that the luster which characterizes the living animal entirely disappeared by the bleaching process. As this luster, which has its seat in the epidermis, depends on the interference of light, it is evident that the cells had undergone *some* change, but a change so slight that the tissues could hardly be said to have been injured for histological purposes; besides, the removal of the osmic acid leaves the animal in a good condition for staining.

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[1] One of the best objects for testing methods is found in *Phronima sedentaria.* Here the cells and nuclei are so sharply defined that they can be seen in the living animal, and so the effect of a preservative fluid can be easily studied.

[2] A slightly modified form of the method originally given in Müll. Arch., 1874, p. 321.

[3] Nitric acid may be used instead of HCl.

Dr. Mayer's experience with Sapphirina appears to support him in the following conclusions in regard to the nature of the action of osmic acid, viz., that the hardening effect of the acid is due to the formation of inorganic precipitates within the tissues. This is made evident by the fact that the animal becomes soft and flexible as soon as these precipitates are removed by bleaching.

This method of bleaching has been used by Dr. Mayer for removing natural pigment. Alcoholic preparations of the eye of Mysis, for instance, can be fully bleached in toto, but with better success by operating with single sections. To avoid swelling, which is apt to arise by the use of aqueous fluids, staining media of an alcoholic nature should be used.

6. *Chromic Acid.*—Chromic solutions have in common with osmic acid, the peculiarity of hardening by virtue of the chemical combinations which they form with cell-substances, and all the consequent disadvantages with respect to staining. The use of chromic acid in the Zoölogical Station of Naples may be said to have been largely superceded by *picro-sulphuric acid*, *corrosive sublimate* and *Merkel's fluid*, for it is now seldom used except in combination with other fluids.[1] It is sometimes mixed with Kleinenberg's fluid, for example, when a higher degree of hardening is required than can be obtained by the use of the latter fluid alone. It is a common error to use too strong solutions of chromic acid, and to allow them to act too long. Good results are in some cases obtained when the objects are treated with a weak solution (⅓–½ per cent.) and removed soon after they are completely dead.

7. *Merkel's Fluid.*—

Platinum chloride dissolved in water	....	1:400
Chromic acid " "	....	1:400

Professor Merkel,[2] who employed a mixture of these two solutions in equal parts for the *retina*, states that he allowed from three to four days for the action of the fluid. Dr. Eisig has used this fluid with great success in preparing the delicate lateral organs of the Capitellidæ for sections, and recommends it strongly for other annelids. Dr. Eisig allows objects to remain 3–5 hours in the fluid, then transfers to 70 per cent. alcohol. With small leeches I have found one hour quite sufficient, and transfer to 50 per cent. alcohol.

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[1] Dr. Pfitzner ("Morph. Jahrb.," B. XVII, p. 731, 1882) has recently made use of chromic acid followed by (1) *osmic acid*, or by (2) *chloride of gold, formic acid* and *safranin* (or hæmatoxylin) for the demonstration of nerve-terminations.

Flemming (see his method on a following page) believes that chromic acid is one of the most reliable fixing reagents for the karyakinetic figures, and has proved that objects hardened in this acid can be beautifully and durably stained.

[2] "Ueber die *Macula lutea* des Menschen," &c., Leipzig, 1870, p. 19.

8. *Corrosive Sublimate.*—Prompted by a statement found in an old paper by Blanchard,[1] Dr. Lang began experimenting with corrosive sublimate as a medium for killing marine Planarians, and his marked success led him and others to employ the same with other animals. In most cases Dr. Lang now uses a *saturated solution of corrosive sublimate in water.* A saturated solution in picro-sulphuric acid, which in some cases gives better results if a little acetic acid (5 per cent. or less) is added, is also used.[2] Blanchard's mode of treatment was to mix a quantity of the aqueous solution with the sea water, and thus poison the animals. Dr. Lang, on the contrary, removes the sea water so far as possible before applying the solution. With Planarians he proceeds in the following manner:

The animal is laid on its back and the water removed with a pipette, the solution being then poured over it, it dies quickly and remains fully extended. After half an hour it is washed by placing it in water and changing the water several times during thirty minutes. It is next passed through 50 per cent., 70 per cent., 90 per cent. and 100 per cent. alcohol. In two days it is fully hardened, and should then be stained and imbedded in paraffin as early as possible, as it is liable to become brittle if left long in alcohol. The time required by the corrosive sublimate varies with different objects, according to size and the character of the tissues. As a general rule, it may be said that objects should be removed from the fluid as soon as they have become thoroughly

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[1] "Recherches sur l'Organisation des Vers," by Emile Blanchard. Ann. des Sci. Nat. Zool. Ser. 3, t. VIII, 1847, p. 247.

[2] These solutions given in *Zoolog. Anzeiger*, 1879, II, p. 46.

The original solution (*Zoolog. Anzeiger*, 1878, I, p. 14–15), now little used, stood thus:

Distilled water	100	parts.
Common salt	6–10	"
Acetic acid	5–8	"
Corrosive sublimate	3–12	"
Alum (in some cases)	½	"

saturated by it. In order to kill more quickly than can sometimes be done at the ordinary temperature, the solution is heated, and in very difficult cases may be used boiling.

Corrosive sublimate has been used with success by Dr. Lang and others in the following cases: Hydroids, corals, Nemertines, Gephyrea, Balanoglossus, Echinoderms, Sagitta, Annelids, Rhabdocœla, Dendrocœla, Cestodes, Trematodes, embryos and adult tissues of Vertebrates and, according to Mayer and Giesbrecht, Crustacea with thin chitinous envelopes, *e. g.*, Sapphirina, Copepods and larvæ of Decapods.

The two great advantages of Dr. Lang's method are, (1) that animals so treated are easily stained, and (2) they are killed so quickly that they are left, in most cases, in a fully extended condition. Hot corrosive sublimate kills leeches so instantaneously that they often remain in the attitude assumed the moment before the fluid is poured over them. The color, however, is not so well preserved as when killed with alcohol, or even with weak chromic acid.

It should be remembered that objects lying in a solution of corrosive sublimate must not be touched with iron or steel instruments; wood, glass or platinum may be used.

9. *Dr. Andres' Methods of treating Actiniæ.*—Among the various methods employed by Dr. Andres in killing the Actiniæ, the three following, given in the order of their excellence, are said to have worked most satisfactorily:

A. Corrosive sublimate.—With small animals a hot solution, used in the manner recommended by Dr. Lang, gives good results; with larger animals, where this mode of treatment fails, the fluid must be injected. The cannula of a glass syringe, filled with the hot fluid, is inserted into the mouth at the moment it opens, which act habitually follows on gently touching the lip. After injecting, the hot solution is poured into the glass containing the animal and a small quantity of sea water.[1]

If the operation is cleverly performed, the animal remains fully expanded, as the mechanical pressure of the injected fluid prevents contraction.

After from five to fifteen minutes the animal is washed in distilled water and allowed to remain twelve hours in 50 per cent.

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[1] *Andres.* "Intorno all'Edwardsia Claparedii," in the Proceedings of the "Reale Accademia dei Lincei," Vol. v, Ser. 3, Mar. 7, 1880, p. 9.

alcohol,[1] then passed through the higher grades of alcohol. Borax-carmine and hæmatoxylin used for staining.

B. Glycerine and Alcohol.[2]—

Glycerine	20 parts.
Alcohol (70 per cent.)	40 "
Sea water	40 "

This mixture, poured very slowly into the containing glass, often gives very good results, both for anatomical and histological purposes.

C. Nicotine and Tobacco Smoke.—*a.* A solution of nicotine (1 g.) in sea water (1 l.), conducted into the vessel containing the animal fully expanded in a half liter of sea water, by means of a thread sufficiently large to empty the flask holding the nicotine solution in the course of twelve hours.

b. The vessel containing the animal in an extended condition, covered by a bell jar in which tobacco smoke is confined, until the animal becomes completely benumbed.

After being deprived of sensibility by either of these methods, the creature may be killed in corrosive sublimate, or in picro-sulphuric acid.

D. Dr. Andres finds that in the use of chloroform, dropped slowly into the water, or administered in form of vapor, maceration usually sets in before the power of contracting is lost. Good preparations of the internal parts may be obtained by injecting a weak solution of osmic acid. The method of freezing has also been employed with some success. For this purpose three vessels are placed one within the other, the central one containing the actinia, the middle one ice and salt, and the outer one cotton. The ice containing the congealed animal is dissolved in alcohol or an acid.

E. Maceration.—It is often important to see the cells of a tissue *in situ* before freeing them with needles. In such cases Dr. Andres proceeds as follows:

1. Killed with corrosive sublimate.
2. Left in 25 per cent. alcohol twenty-four hours.
3. Soaked for a short time in a very thin solution of *gum arabic*, then in a somewhat thicker solution, and finally imbedded in a very thick solution.
4. Hardened in 90 per cent. alcohol.
5. Thick sections prepared for dissection with needles. The sections are placed on a slide in water, which dissolves the gum.

(To be continued.)

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[1] A little camphor (1 ccm. to 100 ccm.) added to the alcohol will facilitate the removal of the sublimate.

[2] This method originated with Salvatore Lobianco.